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13. ABSTRACT ***(Maximum 200 Words)***

Breast cancer is the second leading cause of cancer incidence and the leading cause of cancer mortality in women. Current chemotherapeutic treatments often have adverse effects primarily caused by the inefficient delivery and/or poor specificity of the compounds to breast tissues. Overall, we were interested in determining whether current therapeutic agents be redesigned to carry "tissue specific markers" to enhance their delivery and uptake in targeted tumor cells. In order to accomplish this goal, efficient delivery and increased specificity of chemotherapeutic agents to targeted cells must be achieved. Recently, researchers identified germline mutations in the tumor suppressor gene *BRCA1* that predisposes women to early onset breast cancer. To recapitulate this condition, our laboratory generated a Brca1 mouse model that selectively develops mammary tumors between 6-9 months of age. Using this Brca1 breast cancer model and cell lines derived from mammary tumors, phage display was used to isolate and identify peptide motifs that selectively bind to cultured Brca1 mammary tumor cell lines. Next, the identified candidate peptides were conjugated to a tracer and tested for *in vitro* and *in vivo* efficiency and specificity of delivery toward mammary tumor cell lines and mammary tissues.

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Introduction

Breast cancer is the most common cancer and the second leading cause of cancer mortality in women. Mutations in BRCA1 have been identified in 50 and 80 percent of familial cases of breast or breast/ovarian cancer, respectively. To gain further insite into this devastating disease, our laboratory has recently generated a mouse model for BRCA1 familial cancer with a unique disruption of BRCA1 in mammary epithelial cells (1). Tumorigenesis occurs at a low frequency after long latency, suggesting that multiple factors may be involved in BRCA1 related tumorigenesis. In addition to studying pathways dysregulated in hereditary breast cancer, our laboratory is also utilizing these mice as pre-clinical models to carry out novel chemoprevention studies.

The currently available chemotherapeutic treatments often have adverse effects primarily caused by the inefficient delivery and/or poor specificity of the compounds to breast tissues. Untoward effects such as myelosuppression and dose dependent cardiotoxicity are often associated with common chemotheraputic regimens. Many methods are currently under investigation to circumvent the side effects of systemic application of theraputic agents. Recently, novel techniques such as protein, gene, and lyposomal therapies have been investigated as alternative delivery approaches for theraputic agents. However, each method lacks efficiency and specificity for targeted tissues. Overall, we were interested in determining whether current therapeutic agents be redesigned to carry "tissue specific markers" to enhance their delivery and uptake in targeted tumor cells [Figure 1A]. In order to accomplish this goal, efficient delivery and increased specificity of chemotherapeutic agents to targeted cells must be achieved.

Recent data suggests tissue specific peptide motifs can be identified using phage display and biopanning (2). To identify tissue specific markers we used phage display, a selection technique where random peptides (7-mers) are incorporated into the genome of bacteriophage and the random peptides expressed as part of the bacteriophage virion coat.

In its simplest form, biopanning is carried out by incubating a library of phage-displayed peptides with your target of interest, washing away the unbound phage, eluting the specifically-bound phage, and amplification. Enrichment of the cell associated phage is carried out with additional rounds of binding/amplification cycles which selects or favors sequences that selectively bind to the target [Figure 1B]. The resulting phage are sequenced and tested for binding.

Using this Brca1 breast cancer model and cell lines derived from mammary tumors, phage display was used to isolate and identify peptide motifs that selectively bind to cultured Brca1 mammary tumor cell lines. Next, the identified candidate peptides were conjugated to a tracer and tested for *in vitro* and *in vivo* efficiency and specificity of delivery toward mammary tumor cell lines and mammary tissues.

Body

1. Candidate peptide selection.

To initiate this study, biopanning was performed to select peptides that bind Brca1 mammary tumor cell lines and fibroblasts, as controls. After the first round of biopanning, 3.4 x 10^6 colony forming units (CFUs) were recovered from the panned Brca1 cells and 1.65 x10^6 CFUs from fibroblasts that serve as biopanning controls [Figure 1C]. Two more rounds of phage amplification and biopanning selection resulted in the recovery of 13 x10^6 and 7.8 x 10^6 CFUs for the Brca1 cells and fibroblasts, respectively [Figure 1C]. Therefore, increased numbers of recovered CFUs with successive rounds of biopanning and amplification suggests selective amplification of phage that bind to cells of interest.

Next, 100 clones from the Brca1 display library were amplified and sequenced to initiate the screen and to determine whether the clones showed overlapping amino acid motifs. After data analysis, there were at least 6 recognizable amino acid motifs [Figure

1D]. None of the amino acid motifs or peptides identified in our Brca1 phage library was found in our control fibroblast display library, which was performed simultaneously with our Brca1 screening, or random clones isolated from our initial titer. These results suggest that the peptides may be selective for Brca1 tumor cells.

2. In vitro specificity and efficiency of candidate peptide delivery.

Next, we wanted to determine which of the peptides identified in our Brca1 phage library was specific for Brca1 tumor cells. In this set of experiments, the question was asked whether the identified candidate phage demonstrate uptake and specificity *in vitro*. Therefore, each of the amplified and isolated phage clones from the Brca1 display library was incubated with Brca1 tumor cells and fibroblasts to determine which amino acid motif and peptides were selective for Brca1 tumor cells [Figure 2A]. As shown in Figures 2B and C, phage clones A and B from our Brca1 display library also bound to our control fibroblasts which suggests that these phage clones can bind to a variety of tissues. However, many of the Brca1 display library clones (clones C-L) were more selective for Brca1 tumor cells [Figures 2B,C]. These results suggested that candidate peptides carrying the S/GLP amino acid motif would be the best test candidates for further characterization.

Next, we were interested in determining the most effective marker or tracer for our peptide delivery system. Here, we wanted demonstrate that the peptides, as opposed to the original phage, could bind to cells. This could only be accomplished by identifying a tag that could be added to the peptides so they may be traced in our *in vitro* studies. Our selection of FITC as a tracer or marker, however never demonstrated uptake of FITC tagged peptides *in vitro*. This result could suggest that 1) the isolated phage clones do not bind to the cells. 2) the peptides derived from the phage library require the 3-dimentional structure or additional sequences from the phage coat protein for binding

[Figure 3A]. 3) a linker is required to allow sufficient distance between the tracer and the peptide sequence for cell binding. 4) the hydrophobic nature of FITC prevents peptide binding to live cells.

In order to resolve some of the issues and demonstrate our Brca1 phage display library indeed identified peptides that were selective for our panned Brca1 tumor cells, we modified our tracer. Instead of using FITC as a tracer, we generated peptides that incorporated the HA tag into our peptide sequence. Several characterized tags with available antibodies could have been investigated, however the HA tag contained fewer negative charged residues which could potentially be repelled by the living cells [Figure 3B]. In addition, the length of linkers was also tested [Figure 3C].

Next, we were interested in determining whether our new tracer strategy indeed would accomplish our goals. Therefore, we incubated the HA-S/GLP-peptides with our cell lines. As shown in Figure 3D, our candidate peptides (HA-S/GLP-peptides) carrying the HA tag did not bind to fibroblasts. However, when the peptides were incubated with Brca1 tumor cells, we found uptake of the peptides which demonstrates that our tag system would be an excellent means to identify cells that are bound by our Brca1 peptides [Figure 3E and 3G].

3. *In vivo* specificity and efficiency of candidate peptide delivery.

Next, we ask whether our candidate peptides have specificity for targeting cells *in vivo*. Cell lines generated from our Brca1 mammary tumors were injected into nude mice. After tumors developed we injected our candidate peptides and/or controls into these mice and performed immunohistochemical analysis on a variety of tissues and tumors to determine the delivery of our peptides. In addition, mice with palpable mammary tumors were injected with our tagged targeted peptides. As shown in Figure, immunohistochemical analysis of normal appearing mammary [Figure 4B] and mammary

tumors [Figure 4C] of mice injected with our tagged peptides demonstrated binding in regions of mammary epithelial cells. However, upon further analysis of other tissues from these mice suggests that we were detecting our tagged peptides that are retained in the blood stream, primarily bound to red blood cells [Figure 4D]. This data suggests that further investigations are required to determine methods of preventing binding of our peptides to serum cells/proteins while allowing the peptides access to mammary tumor cells. The best method of accomplishing these goals would probably be with combined use of our peptide system in addition to polymers or sterically stabilized liposomes.

4. *In vivo* specificity and sensitivity of candidate peptides carrying therapeutic agents.

In this series of experiments, we wanted to determine whether the candidate peptides conjugated to Doxorubicin (Adriamycin) has the ability to inhibit tumor growth. First we were interested in determining whether Doxorubicin had the ability to kill our Brca1 breast tumor cells. As shown in Figure 4E, our Doxorubicin dose curve demonstrates that over 50% of our cultured Brca1 tumor cells do not survive at 0.1 μg/ml. This data suggests that when we have overcome the non-specific binding of our peptide to serum red blood cells, that Doxorubicin may be an excellent candidate for our future *in vivo* studies.

Key research accomplishments

Overall, this study provided an investigation of using phage display as a means of identifying peptides that could be used as a means of selectively targeting drugs to tumors. In this study, we identified specific motifs that bound to Brca1 tumor cells [Figure 1D]. Second, we demonstrated the specificity of these motifs for Brca1 tumor cells [Figure 2B and C]. Lastly, we identified the appropriate "tag" system that could be

used to trace the *in vitro* [Figure 3] and *in vivo* [Figure 4] distribution of our candidate peptides.

Reportable outcomes

We currently have a publication in press where the response of our Brca1 tumor cell lines to various chemotherapeutic agents is reported. This data suggests that the Brca1 tumor cell lines are Tamoxifen resistant but highly sensitive to Doxorubicin or γ-irradiation, demonstrating that Doxorubicin would be the best chemotherapeutic candidate for future targeting studies.

Brodie S.G., Xu, X., Qiao W., Li. W., Cao L., Deng, C.X., (2001). Multiple genetic changes are associated with mammary tumorigenesis in Brca1 conditional knockout mice. (In Press; **Oncogene**).

Conclusions

Our goal was to develop a highly specific and efficient method to deliver drugs to tumors. In this series of experiments we were interested in determining whether phage display could be used to identify peptides that could act as a beacon to target chemotheraputic agents to tumor cells. This could effectively increase the local dose of the drug in tumors. The results of this study suggests that this technique is viable and allowed the identification of candidates that could be tested both *in vivo* and *in vitro.* We also identified the linker length and identified the "tag" that could be conjugated to the peptides for tracing our carrier molecules *in vitro* and *in vivo.* We also found that further studies are required to prevent the nonspecific binding of our peptides to serum components. Upon accomplishment of these future experiments we will be able to determine whether targeted delivery of therapeutic agents is viable as a breast cancer treatment. Moreover, if targeted therapeutic treatment increases the efficacy and

specificity of chemotheraputic agents then perhaps this concept may lead to treatments for other forms of cancer.

References

1) Xu, X. et al. (1999) Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat Genet 22, 37-43.

2) Arap W. et al (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279, 377-380.

Appendices

Figure 1. Phage display and biopanning used for candidate peptide selection.
(**A**) Diagram of the overall goal of the proposed research. (**B**) Diagram showing the technique of biopanning. (**C**) Graph demonstrating the numbers of phage recovered (CFU's) with sucessive round of biopanning. (**D**) Peptide motifs and sequences identifed from the Brca1 display library.

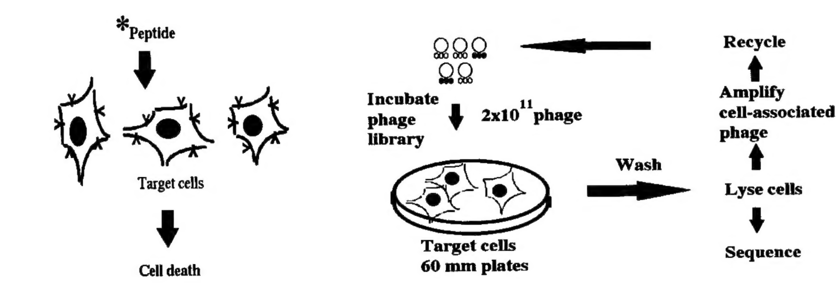
Figure 2. In vitro specificity and efficiency of Brca1 display phage.
(**A**) Diagram of the overall goal invitro specificity and efficiency of candidate phage. (**B**) Plate showing the means of identifying colony forming units (CFU's). (**C**) Graph demonstrating the numbers of phage recovered (CFU's) from specificity assay.

Figure 3. In vitro specificity and efficiency of candidate peptide and tag delivery.
(**A**) Protein sequence of phage coat protein surrounding the peptides. (**B**) Peptide sequences of various "tags" which could be utilized. (**C**) Dipection of our candidate constructs showing our peptide-linker-tag sequence. (**D-G**) Immunohistochemical staining for our candidate peptides incubated with fibroblasts (**D**), Brca1 cells (**E and G**). (**F**) Negative control

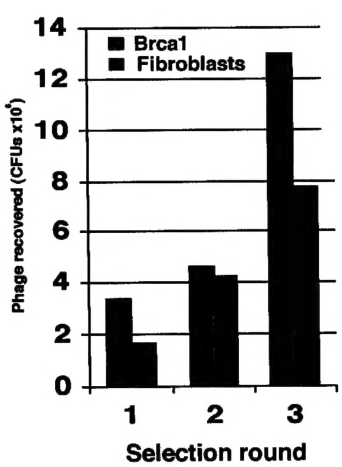
Figure 4. In vivo specificity and efficiency of tagged candidate peptides.
(**A-D**) Immunohistochemical staining for our candidate peptide after injection into Brca1 mammary tumor mice. Arrows point to positive staining in mammary tissue (B), mammary tumor (**C**) and Liver (**D**). (**A**) Negative control.

1A

1B



1C



1D

MOTIF	Peptide sequence	PEPTIDE
SSAXL	ASHSSAPL	A
	SSALLSR	G
P/VPP	VPPQLFG	B
	VPPRLPV	K
S/GLP	VPSLPFP	C
	SLPIATR	E
	QNNSLPF	F
	GLPPPQR	H
	GLPTHQL	D
KQ	GFGSKQT	I
	AKRTKQY	J
SYX	SYVQYPH	L

Figure 1.

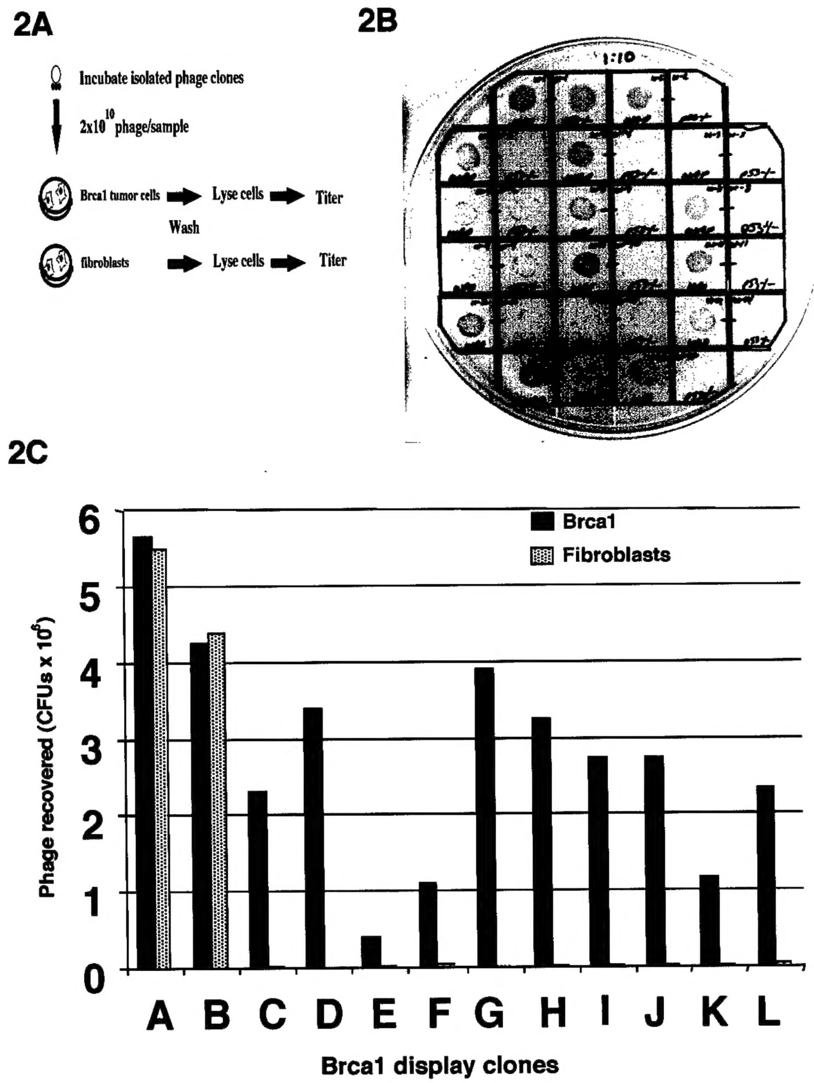

2A
Incubate isolated phage clones
2x10^10 phage/sample
Brca1 tumor cells
Lyse cells
Titer
Wash
fibroblasts
Lyse cells
Titer
2B
1:10
2C
Brca1
Fibroblasts
Phage recovered (CFUs x 10^6)
0
1
2
3
4
5
6
A B C D E F G H I J K L
Brca1 display clones

Figure 2.

3A .....PFYSHS-XXXXXXX-GGSA.....

3B

Tag	Peptide sequence
Flag	MDYKDDDDK
c-Myc	MEQKLISEEDL
HA	YPYDVPDYASL

3C

Peptide Linker HA

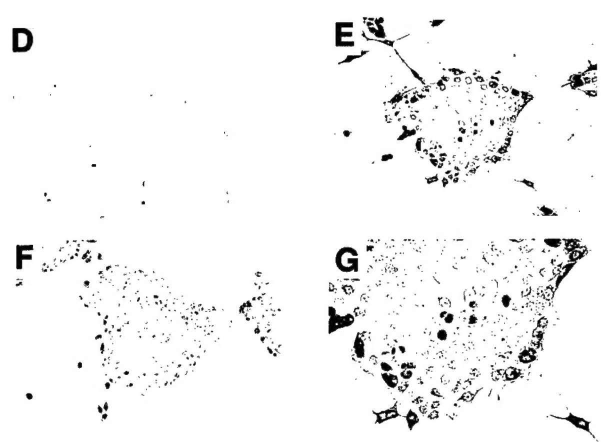


Figure 3.

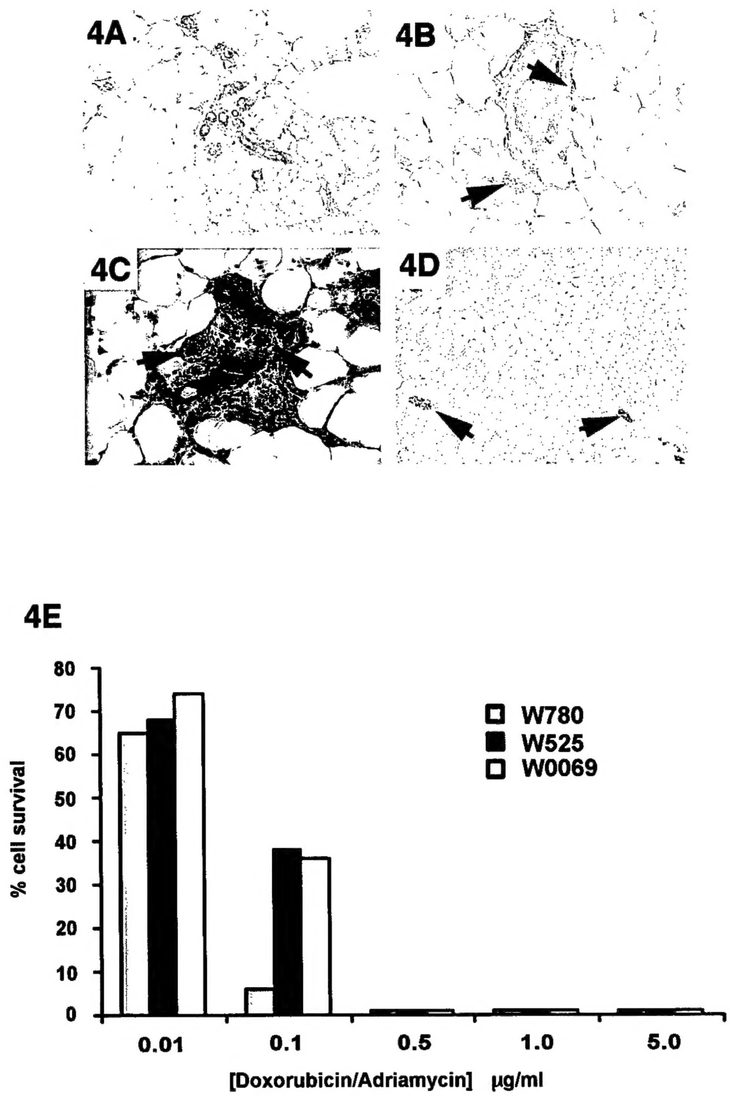

4A
4B
4C
4D
4E
80
70
60
50
40
30
20
10
0
% cell survival
W780
W525
W0069
0.01
0.1
0.5
1.0
5.0
[Doxorubicin/Adriamycin] µg/ml

Figure 4.